Reference: *Biol. Bull.*, **136**: 434–453. (June, 1969)

DEVELOPMENT OF ARTIFICIAL MEDIA FOR *ARTEMIA SALINA*[1]

LUIGI PROVASOLI AND ANTHONY D'AGOSTINO

Haskins Laboratories, 305 E. 43rd St., New York, New York 10017 and Department of Biology, St. Johns University, Jamaica, New York 11432

Much is known about the nutritional requirements of Insecta. This reflects progress in devising artificial media which meet the physical requirements of their varied types of feeding. Phytophagous, saprophagous, and parasitic insects have been fed on media brought to suitable consistency by agar or other solidifiers. Blood or plant-sap sucking insects present physical problems partly solved by feeding them on liquid media contained in membranes which can be pierced by the sucking organs. Little progress has been made with artificial media for filter-feeders. Yet such feeding is used by most aquatic invertebrates from sponges to arthropods, even by primitive chordates (*e.g.* sea squirts).

Filter-feeders are ostensibly phagotrophic but is phagotrophy obligate or facultative, *i.e.*, can the nutrient particles be replaced in part or totally by solutes? So far some mosquitos whose larvae are filter feeders were grown axenically; *Aedes aegypti* has been grown in chemically defined media, (Singh and Brown, 1957; Akov, 1962).

Artemia salina was selected for the present work because its eggs are commercially available, easy to purify and, because it tolerates a wide range of salinities, it might withstand high concentrations of organic and inorganic substances. A complex artificial medium capable of supporting growth from nauplii to adults had been devised (Provasoli and Shiraishi, 1959).

We describe here a simpler medium for *Artemia* and some nutritional problems presented by filter-feeders.

Methods and Results

The bisexual tetraploid race of *A. salina* from the Great Salt Lake (Utah strain) was used throughout. Techniques for axenizing eggs, culturing nauplii, and testing sterility of inocula remained unchanged (Provasoli and Shiraishi, 1959).

Two methods were used to assess the adequacy of the media: *1*) *Serial transfer.* Nauplii (24–48 hr old) were inoculated into experimental media (5 ml) containing soluble (liquid phase) and insoluble nutrients (particulate phase). Surviving or developing organisms were transferred every 5 to 8 days into duplicate tubes (10 ml) until they stopped growing or died; *2*) *Drop-wise feeding.* The nauplii were inoculated into the liquid phase of the artificial media (10 ml) containing only the soluble inorganic and organic components and finely dispersible substances (solubilized fatty acids and sterols). The complete medium containing the particles (insoluble rice starch and heat-precipitated albumin and/or globulins), was added drop-wise to this liquid phase (1 ml aliquots) at inoculation, and every few days,

[1] Aided in part by contract Nonr 4062 with the Office of Naval Research and research grant GB-4860 of the National Science Foundation.

i.e., as soon as the organisms cleared the particulates. When the organisms had become juveniles they were transferred to a 10-ml tube containing the complete medium.

To facilitate graphic comparisons of the experiments, growth was expressed as the number of molts per animal per volume of media (5 or 10 ml) obtained during the first 12–17 days of growth. The first 14 molts, culminating in an adult animal, are easily distinguished because of the changes in appearance. Adult artemias after the 14th molt continue to molt every few days but the morphological changes after each molt are subtle: gradual increase in the size of the claspers (2nd antennae of the male) and the ovisac. Each stage of the life cycle was identified by culturing many neonates to adults, each in a separate container, observing them twice daily, and identifying the instar from the number of exuviae produced and by examination of fixed "typical" animals (D'Agostino, 1965). Heath (1924), in basing the stages on frequencies of appearance in field collections, missed some instars. Precise designation can now be assigned to the working description of the stages recorded previously (Provasoli and Shiraishi, 1959): "metanauplii III small and big" = 3rd to 6th instar; "metanauplii IV "small" to "big" cover the span of the 7th to 11th instar; "juvenile" = 12th instar; "young" the 13th; and male and female, from the 14th instar on (Fig. 1).

An arbitrary method was devised to express growth numerically. It was difficult to measure growth because generally (in poorly balanced media) the 5 animals inoculated in one tube had different growth potentials. A similar variability was observed in the parthenogenetic strain of *A. salina* collected in Comacchio (Italy) by Dr. Barigozzi, even after several hundred parthenogenetic generations derived from the original single female. Parthenogenetic *Daphnia magna* showed the same phenomenon: 1–2 individuals out of 5 grow much faster. Typically, in 17 days growth in 10 ml of medium, 1 animal was at the 14th instar, 2 animals at the 12th, 1 at the 11th, and 1 at the 7th instar. Comparing nutritional variables thus posed a serious problem. We eventually scored individuals by assigning them an arbitrary value ("growth index") which approximates number of molts and size (Legend Fig. 1, A–I). The sum of these 5 numbers divided by 5 served as index of the total growth in a particular tube (in the above case 13 + 22 + 10 + 5 = 50:5 = 10.

This index served for numerical or graphical comparisons and proved reliable despite its crudeness.

Preparations

Starch particles Insoluble rice starch was ground in a colloidal mill; 100-mg portions were transferred in screwcap test tubes with some glass beads (1 mm diameter) then sterilized in a dry oven at 180° C for 2 hr, allowed to cool, and 10 ml of sterile distilled water were added aseptically (1 ml of suspension = 10 mg starch). This slurry was shaken and added aseptically to the experimental tubes.

Starch gel Insoluble rice starch was suspended in aliquots of medium (proportion 1 g in 40 ml of water), brought to a boil, then cooled. The gelled starch was added to the remainder of the medium and homogenized with a magnetic stirrer. The media were then dispensed and autoclaved. Starch not previously gelled if

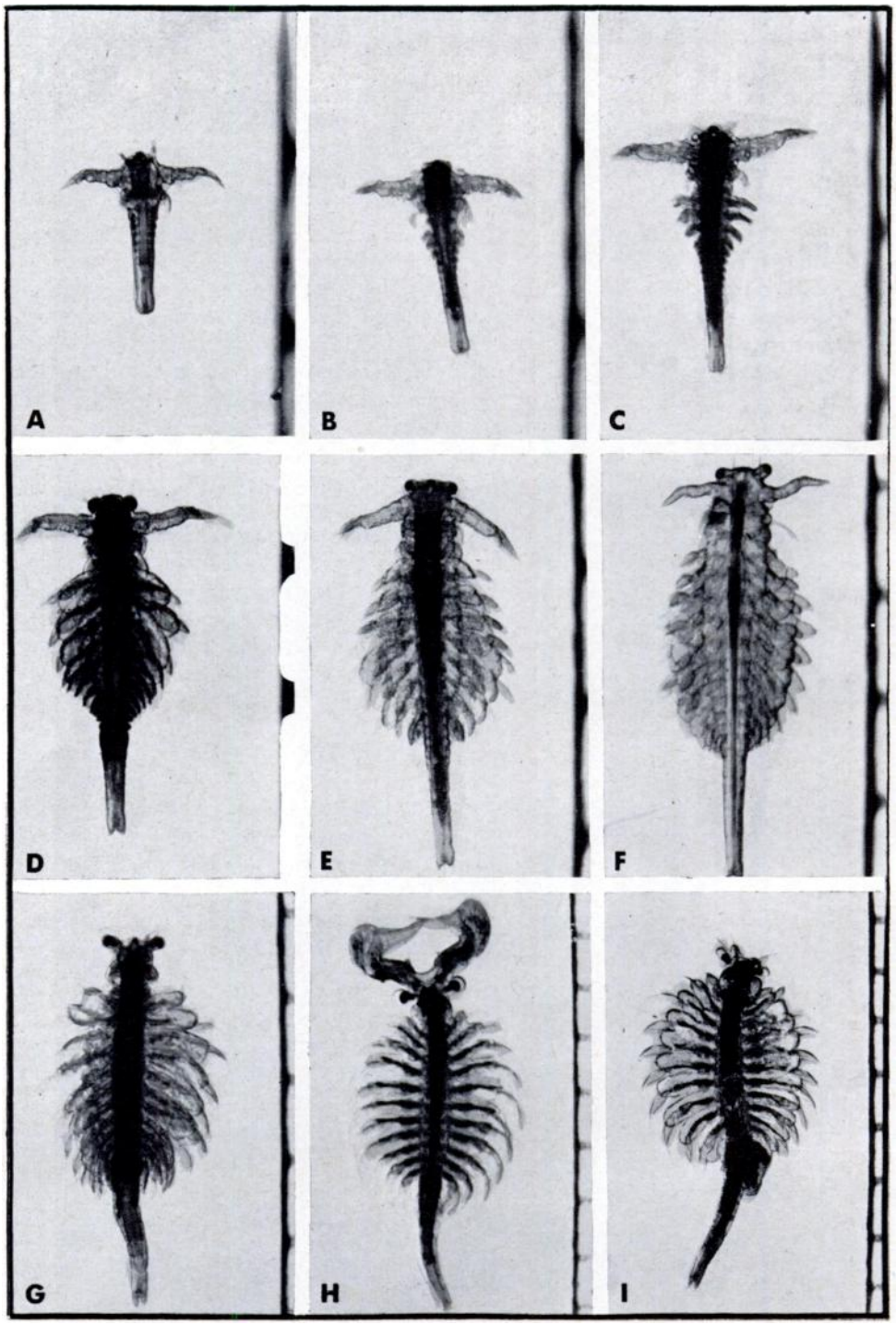

FIGURE 1, A–I.

suspended in media before autoclaving invariably formed aggregates and sometimes foamed out of the tubes during autoclaving.

Various sugars Concentrated stock solutions of soluble sugars were glass filter-sterilized and added aseptically to culture tubes.

Aseptic protein solutions Crystallin egg albumin, γ-globulin, and β-lactoglobulin were dissolved in either seawater or an equivalent salt solution and Seitz-filter sterilized. Such filtrates and commercial aseptic serum were added aseptically to culture tubes.

Protein particles One g of crystalline proteins (egg albumin, γ-globulin and/or β-lactoglobulin) was dissolved in 50 ml of seawater. The dissolved protein was transferred to a Virtis homogenizer; 10 g of glass beads were added (25 μ diameter; "Super Brite," Minnesota Mining and Manufacturing Co.). The solution was slowly brought to a boil and heated until protein precipitation was virtually complete. The precipitate was homogenized and autoclaved at 20 lb for 30 min. During autoclaving more precipitate formed; the coagulum was rehomogenized. Sufficient slightly buffered (bicarbonate) distilled water (pH 7–8) was added to resuspend the precipitate. The suspension was decanted into a stainless-steel sieve (0.038 mm pore size, Newark Wire Cloth Co., Newark, New Jersey) which retained the glass beads. The volume of the filtrate containing the fine suspended protein particles was readjusted to 50 ml by boiling off excess water. The final suspension contained 20 mg of protein per ml. This preparation was added to the final medium before autoclaving (15 lb, 20 min). The procedure was later modified to eliminate possible chemical contaminations: an artificial seawater was used; the glass-beads step was replaced with a more efficient high-speed 4-bladed homogenizer. This eliminated addition of the excess water needed for filtering off the glass beads and the subsequent boiling off of excess water (which may have caused unwanted hydrolysis of protein).

Liver Oxoid L-25 This water-soluble extract of beef liver (Colab Labs. Inc., Chicago Heights, Illinois) was used to supplement media with unidentified growth factors. It was added to media before autoclaving; during autoclaving a little brown precipitate forms.

Co-gels Mixtures of protein and starch were co-gelled to provide small particles containing both. This was done to favor growth of naupliar stages (especially of *Tigriopus*). Very fine particles are needed because of their small food-gathering parts. Since the gut of nauplii is small, the co-gel avoided excess feeding on either starch or proteins; selection of particles depending on size.

The proportions starch:albumin could be varied at will, but the proportion of solute to solvent had to be kept within narrow limits in order to obtain a firm gel

FIGURE 1. Developmental stages of *A. salina*. A. Metanauplius III small = 3rd instar; 0.9–1 mm long; 24–48 hr. old; employed generally as inoculum. B. Metanauplius III big = 5, 6 instars; 1.3–1.5 mm long; growth index 4; terminal stage of growth if the medium is incomplete. C. Metanauplius IV small = 7, 8 instars; 1.6–1.9 mm long; growth index 5. D. Metanauplius IV medium = 9, 10th instars; 2.2–2.4 mm long; growth index 8. E. Metanauplius IV big = 11th instar; 2.4–2.6 mm long; growth index 10. F. juvenile = 12th instar; 2.7–4.0 mm long; growth index 11. G. young = 13th instar; 3.5–5 mm long; growth index 12. H. functional male = 19th instar; 6.5 mm long. A male ranges from 5 mm (14th instar, growth index 13) to 9 mm (25th instar). I. mature female = 15th instar; 6.5 mm; growth index 15. Females may reach 11 mm at 24th instar. (The scale is in millimeters.)

(~1:10–20 w/v). Typically, half a gram of insoluble rice starch was mixed well in a solution of crystalline egg albumin (0.1 g in 5 ml H_2O at pH 7.0), giving a ratio starch:albumin 5:1. The colloidal-suspension was slowly heated to a boil while continuously stirred. The gel was allowed to cool. Ten ml of water were added (pH 7.0), and the gel was homogenized, autoclaved at 20 lb for 30 min, cooled, and rehomogenized aseptically until finely suspended (milky consistency). It was transferred to sterile rubber-lined screwcap tubes. This preparation could neither be re-autoclaved nor stored in the refrigerator because the particle would re-aggregate; it remained stable for 15–30 days at room temperature.

The optimal ratio starch per protein seems to vary: for *Artemia* is 5–3:1 while *Daphnia* prefers 1:1 and tolerates well even higher ratios of proteins.

Cholesterol Twenty mg of cholesterol dissolved in 2 ml of 95% ethanol, were squirted with a small-bore needle into 20 ml of boiling water; a fine precipitate formed. The alcohol was evaporated in a water bath and the final volume was adjusted to 20 ml (1 ml suspension = 1 mg cholesterol). Aliquots of this suspension were added to media before sterilization. *Artemia* can withstand 1 ml/100 ethanol but *Daphnia* are sensitive to traces.

Cholesterol was sometimes used dissolved in warm propylene glycol and it was added as such to the media; 10–30 mg% of propylene glycol were non-toxic to *Artemia* or *Daphnia*.

Solubilized fat mixtures Several mixes proved useful and in most cases non-toxic for *Artemia*. A typical mixture, PFTC, was prepared as follows: *1*) *Phospholipid-fat preparation ("PF1")*. One g refined lecithin (Gliddex I, Central Supply Co., Chicago) was dissolved by mixing it thoroughly with 2 grams each of Tween 60 and 80, then 20 ml of propylene glycol (50% in water) were added. The whole was brought to 100 ml with H_2O; it should be clear. *2*) *Phospholipid-fat-cholesterol-taurocholate preparation ("PFTC")*. To 5 ml PF1 were added 3.5 ml of an alcoholic solution of cholesterol (10 mg/ml). After thorough mixing, the alcohol was boiled off, then 15 ml of a Na taurocholate solution (10 mg/ml) were added, brought to a boil and the volume adjusted to 50 ml with H_2O. This also should be a clear solution; 1 ml contained 10 mg propylene glycol, 2 mg each of Tween 60 and 80, 1 mg lecithin, 3 mg Na taurocholate, and 700 μg cholesterol. Used for *Artemia* at 1 ml% (V/V).

Refining the 1959 medium

The original medium was obtained empirically adding rich organic crudes to improve growth (medium "1959" Table I; Provasoli and Shiraishi, 1959). We assumed that particles were needed—starch had been selected, being relatively pure—and that cholesterol, nucleic acid components, and vitamins might be needed by the crustacea much as for fellow arthropods, the insects. Adults were finally obtained by the addition of serum, glutathione, "paramecium factor" (Lilly and Klosek, 1960) and higher vitamin levels; each of these additions improved growth but none dramatically.

The technique was to inoculate five 24–48 hr-old nauplii in 5 ml of the medium. Every 7 days they were transferred to new media (10 ml) whether or not they had consumed all the particles. On the average they became "metanauplii IV big"

TABLE I

Initial medium "1959" (% w.v/v)		Final medium "100" (% w.v/v)	
		NaCl	2.4 g
		$MgSO_4 \cdot 7H_2O$	0.6 g
Seawater	80 ml	$MgCl_2 \cdot H_2O$	0.4 g
H_2O	15 ml	$CaCl_2 \cdot 6H_2O$	0.22 g
Soil extract	5 ml	KCl	60 mg
		Metal mix PII[e]	3 ml
		Metal mix SII[f]	1 ml
		Fe (as Cl)	0.05 mg
K_2HPO_4	1 mg	Na_2 glycerophosphate	50 mg
		Adenylic acid	60 mg
Alk. hydr. RNA[a]	40 mg	Guanylic acid	2.5 mg
Acid hydr. DNA[b]	10 mg	Cytidylic acid	5.0 mg
		Uridylic acid	5.0 mg
		Thymidine	2.5 mg
Liver infusion	100 mg		
Horse serum	5 ml	Egg albumin **P**	10–20 mg
Trypticase BBL	320 mg	L-Threonine*	20 mg
Paramecium factor[c]	5 mg	L-Histidine*	20 mg
Yeast autol. Difco	20 mg	L-Phenylalanine*	10 mg
Glycine	10 mg	L-Serine*	40 mg
L-Glutamic ac.	50 mg	L-Glutamic ac.*	100 mg
DL-Alanine	10 mg		
		Glucose	300 mg
Sucrose	300 mg	Sucrose	300 mg
Rice starch **P**	500 mg	Rice starch **P**	100 mg
Cholesterol **P**	200 μg	Cholesterol **P**	800 μg
Glutathione	30 mg		
Ascorbic ac.	3 mg	Vitamin mix A.VIII[g]	2 ml
Vitamin mix A.II[d]	1 ml	Glycylglycine	100 mg
pH = 7.4		pH = 7.4	

[a] Yeast RNA brought to pH 9.0 with NaOH and steamed for 1 hour.

[b] Herring sperm DNA brought to pH 1.5–2.5 with H_2SO_4 and steamed for 2 hours.

[c] Kindly supplied by Dr. Daniel M. Lilly; see Lilly and Klosek, 1961.

[d] 1 ml Vitamin mix AII contains: thiamine HCl 0.1 mg; biotin 5 μg; folic acid 70 μg; nicotinic acid 0.5 mg; choline 5 mg; Ca pantothenate 0.7 mg; pyridoxine HCl 80 μg; carnitine 0.2 mg; riboflavin 1 μg.

[e] 1 ml metals PII contains: Na_2 EDTA 1 mg; Fe (as Cl) 0.01 mg; B (as H_3BO_3) 0.2 mg; Mn (as Cl) 0.04 mg; Zn (as Cl) 5 μg; Co (as Cl) 1 μg.

[f] 1 ml metals SII contains: Br (as Na) 1 mg; Sr (as Cl) 0.2 mg; Rb (as Cl) 0.02 mg; Li (as Cl) 0.02 mg; Mo (as Na salt) 0.05 mg; I (as K) 1 μg.

[g] 1 ml of Vitamin mix A.VIII contains: thiamine HCl 1.2 mg; nicotinic acid 2.4 mg; Ca pantothenate 4 mg; pyridoxine HCl 100 μg; riboflavin 300 μg; folic acid 0.7 mg; biotin 60 μg; putrescine 200 μg.

P = particles.

* = components of amino acid mixture AA 1A (3.3 ml, see Table 3).

(11th instar) in 12 days, "young" in 15, "young adults" (14th instar) in 20, and full-grown adults in 37 days.

The liquid part of this medium (*i.e.*, without starch) supported no growth beyond the 4th instar (*i.e.*, full resorption of yolk and beginning feeding) indicating that the ingestion of liquids might not suffice to support growth; it was assumed that the starch particles either increased inbibition or might have absorbed the soluble nutrients, making them available by phagotrophy.

Replacement of complex substances Table II gives the compositions of 3 typical basic media which were developed during the stepwise replacement of the crude organic substances with chemically defined or simpler components. Replacement of liver infusion, horse serum, and yeast autolysate was difficult because these preparations contain a great array of nutrients: proteins, amino acids, polysaccharides, vitamins, fats, sterols, and emulsifiers. It was easy to replace Trypticase with an amino-acid mixture. Each time a crude was eliminated the components of the medium had to be rebalanced and often new factors had to be added.

The first successful elimination of serum, yeast autolysate, and liver extract (Table II, medium 31) was by replacing them with *a*) γ-globulin + β lactoglobulin + albumin; *b*) a more complete, far more concentrated mixture of B-vitamins; and *c*) by raising the cholesterol to 300 μg%. However specimens of *Artemia* in medium 31 grew more slowly than in medium 1959 and they generally failed to become sexually mature.

Of all the substances eliminated from the old medium, the liver infusion seemed richest in substances supporting sexual differentiation and oogenesis. However numerous complete amino-acid mixtures and vitamin mixtures failed to stimulate oogenesis even though the amino acids allowed longer survival of adults.

Medium 39 summarizes the efforts at simplification (Table II). Seawater was replaced with an artificial salt solution like the one employed for marine algae (Provasoli, McLaughlin and Droop, 1957). In doing so we found that Tris [(hydroxymethyl)aminomethane] inhibited *Artemia* while glycylglycine was well tolerated at pH-buffering concentrations (*Daphnia* behaved similarly). Glutathione and the "paramecium factor" proved superfluous. In an attempt to eliminate the tedious preparation of filter-sterilized protein solutions, and its risky aseptic addition to all culture tubes, precipitation by heat was tried. Unexpectedly, growth improved. But the addition of more particles seemed inhibitory, consequently the starch was reduced to 0.5% and the total proteins to 0.1%. A new, more concentrated vitamin mixture was used.

The failure to replace liver with amino acids and vitamin mixtures resulted in trying components of liver hitherto disregarded. Crude bile salts at 5–10 mg% replaced 0.05–0.1% of liver infusion. In turn, 3 mg% of Na taurocholate + higher cholesterol (500 μg%) replaced the bile salts. Since bile salts and taurocholate are potent emulsifiers, it was logical to suppose that fat-soluble factors might be involved and that cholesterol might be more effective in emulsion form (it was previously added as an ethanolic solution; after boiling off the alcohol it became a fine precipitate). Several emulsions were tried. Of these, PFTC (see "preparations") proved effective bringing in Tween 60 and 80, [thus providing oleic, palmitic, stearic, and traces of linoleic, and linolenic acids (Shorb and Lund, 1959)], taurocholate, cholesterol, and refined lecithin.

TABLE II

Medium 31 (% w.v/v)		Medium 39 (% w.v/v)		Medium 91 (%. w.v/v)	
		NaCl	3 g	NaCl	2.4 g
		$MgSO_4 \cdot 7H_2O$	0.7 g	$MgSO_4 \cdot 7H_2O$	0.6 g
H_2O	10 ml	$MgCl_2 \cdot 6H_2O$	0.4 g	$MgCl_2 \cdot 6H_2O$	0.4 g
Seawater	85 ml	KCl	0.08 g	KCl	0.06 g
Soil extract	5 ml	$CaCl_2 \cdot 6H_2O$	0.22 g	$CaCl_2 \cdot 6H_2O$	0.22 g
NaCl	0.5 g	Metal mix PII	3 ml	Metal mix PII	3 ml
Metal Mix PII	3 ml	Metal mix S7[a]	0.5 ml	Metal mix S7[a]	0.5 ml
Fe (as Cl)	50 μg	Fe (as Cl)	50 μg	Fe (as Cl)	0.05 mg
		Glycylglycine	0.1 g	Glycylglycine	0.1 g
K_2H PO	10 mg	Na_2glycero $PO_4 \cdot 5H_2O$	10 mg	Na_2glycero $PO_4 \cdot 5H_3O$	50 mg
Alk. hydr. RNA	30 mg	Alk. hydr. RNA	40 mg	Alk. hydr. RNA	80 mg
Acid hydr. DNA	20 mg	Acid hydr. DNA	10 mg	Ac. hydr. DNA	20 mg
Egg albumin	60 mg	Egg albumin **P**	60 mg		
γ-Globulin	60 mg	γ-Globulin **P**	20 mg		
β-Lactoglobulin	80 mg	β-Lactoglobulin **P**	20 mg	Egg Albumin **P**	20 mg
Glycine	10 mg	Aminoacids AAP_1[b]	6 ml	Amino acids AA18L[b]	6 ml
L-Glutamic acid	30 mg	Amino acids AAP_2[b]	1 ml		
DL-Alanine	10 mg				
Amino acids AAP_1[b]	5 ml				
Rice starch **P**	0.7 g	Rice Starch **P**	0.5 g	Rice Starch **P**	0.1 g
Glucose	0.4 g	Sucrose	0.3 g	Sucrose	0.3 g
		Glucose	0.3 g	Glucose	0.3 g
		Propylene glycol*	10 mg		
		Tween 60 + 80 (1:1)*	4 mg		
Cholesterol **P**	0.3 mg	Lecithin*	1 mg	Cholesterol **P**	0.8 mg
		Na taurocholate*	3 mg		
		Cholesterol*	0.7 mg		
Glutathione	30 mg				
		Vitamin mix A.V[d]	1 ml	Vitamin mix A.VIII[e]	2 ml
Vitamin mix A.III[c]	1 ml				
		pH = 7.4			

* components of PFTC mixture, see "preparations."

P = particles.

[a] 1 ml metal mix S7 contains: Br (as Na) 6 mg; B (as H_3BO_3) 0.4 mg; Sr (as Cl) 0.7 mg; Rb (as Cl) 0.01 mg; Li (as Cl) 0.02 mg; I (as K) 5 μg; Mo (as Na salt) 5 μg; F (as Na) 0.1 mg.

[b] see Table III.

[c] 1 ml vitamin mix A.III contains: thiamine HCl 0.17 mg; nicotinic acid 0.5 mg; Ca pantothenate 1 mg; pyridoxine HCl 50 μg; riboflavin 3.5 μg; folic acid 0.2 mg; biotin 15 μg; putrescine 45 μg; inositol 1 mg; choline citrate 1.5 mg; PABA 50 μg; vit. B_{12} 1 μg; carnitine 0.2 mg; orotic acid 0.3 mg; thymine 80 μg.

[d] 1 ml vitamin mix A.V. contains: thiamine HCl 0.3 mg; nicotinic acid 0.7 mg; Ca pantothenate 1.5 mg; pyridoxine HCl 25 μg; riboflavin 10 μg; folic acid 0.25 mg; biotin 20 μg; putrescine 50 μg; inositol 0.3 mg; choline citrate 0.5 mg; PAPA 50 μg; vit. B_{12} 1 μg; folinic acid 10 μg; carnitine 0.2 mg.

[e] see footnote [x], Table I.

Medium 39 was better than medium 31 in all respects and was inferior to the undefined medium "1959" only in that only rarely females produced eggs. *Artemia* in medium 39 developed faster, grew larger, and lived longer than in any other simplified medium and equaled medium 1959. The consumption of media per animal was also considerably reduced. Although sexual differentiation was now normal, oogenesis was induced only by adding 0.05–0.1% liver infusion: liver was supplying further factors.

In Medium 39, and in previous media as well, there was considerable mortality of the inoculum. Often >50% of the nauplii died within 24–48 hr of their transfer from the hatching medium (STP, Provasoli and Shiraishi, 1959) into experimental media. Such mortality necessitated re-inoculation of experimental tubes, increasing the risk of bacterial contamination and the concomitant undesirable addition of more STP, *i.e.*, additional unknown substances. Various factors were found responsible for the high mortality. The Utah *Artemia* nauplii hatch from the wintering eggs with enough yolk to complete 3 molts; therefore exogenous nutrients are not needed during the first 36 hr.

The non-feeding 0–18 hr old nauplii died because the finely suspended particles adhered to the bristles of the second antennae thus impeding swimming. Old nauplii (24–48 hr) were more tolerant but not completely safe from the effects of particles.

Older nauplii (48–72 hr or older) grown in fluid STP were starving and unable to recover especially in suboptimal media.

To inoculate the more resistant older nauplii it was necessary to avoid starvation after they exhausted their yolk. Addition of 4–6 drops of complete medium 39 to 10 ml of STP after the nauplii were 24 hr old was successful: larvae grew rapidly and mortality was negligible (<10%); additional drops of medium 39 were added afterwards as the particles were consumed.

The experimental technique was then changed: *a*) the 24-hr nauplii were inoculated directly into each experimental tube containing only the liquid part of the medium (10 ml), then the particulate medium containing the same nutritional variable was added dropwise (1 ml at a time, as the particles were consumed); nutritional carryover was thus avoided; *b*) 5 nauplii were still inoculated in each tube but growth was scored at 12 or 17 days because by then the results were clear: adults (>14 instar) were often obtained in 15–17 days as opposed to 33 days with the serial transfer system. Important results were checked periodically with replicate experiments. Several requirements were determined with this technique. The vitamin needs were determined by the usual subtraction tests, resulting in vitamin VIII mix (Provasoli and D'Agostino, 1962). It was also found that: higher concentrations of nucleic acid components were needed for faster growth; that cholesterol at a much higher level (0.8 mg%) was the only ingredient in the PFTC mixture needed (taurocholate and bile salts supplied cholesterol), and that albumin was the best protein thus eliminating the globulins.

The dropwise feeding technique, although useful, was not a practical solution as a growth medium: it was laborious and required stringent aseptic techniques to avoid infections during the repeated opening of the tubes. It was then desirable to return to a more conventional procedure. We knew that the total quantity of particles had to be reduced to avoid nauplii mortality but what was the optimal

amount to maintain a high growth rate? This led to inquiries on the role of particles, (see below) and to a practical solution: use of 0.1% starch + 0.03% or 0.02% albumin.

These advances were embodied in medium 91 which served for studies on replacing the particulate starch and precipitated protein with soluble carbon and nitrogen sources and for the determination of the purine-pyrimidine requirements. In Medium 100 (Table I) all nutrients aside from starch and albumin are defined. Mortality in this medium was low (<10%) and adults (>15 instar) were obtained in 23 days after 2 transfer to 10 ml of new medium. Medium 100 still lacks the factors, supplied by liver infusion, for oogenesis. Without the addition of liver extract females carried eggs at 39 days but died at 45 days without depositing the eggs. With the addition of 75 mg% of liver extract females carrying eggs were obtained after 21 days and deposition after 25.

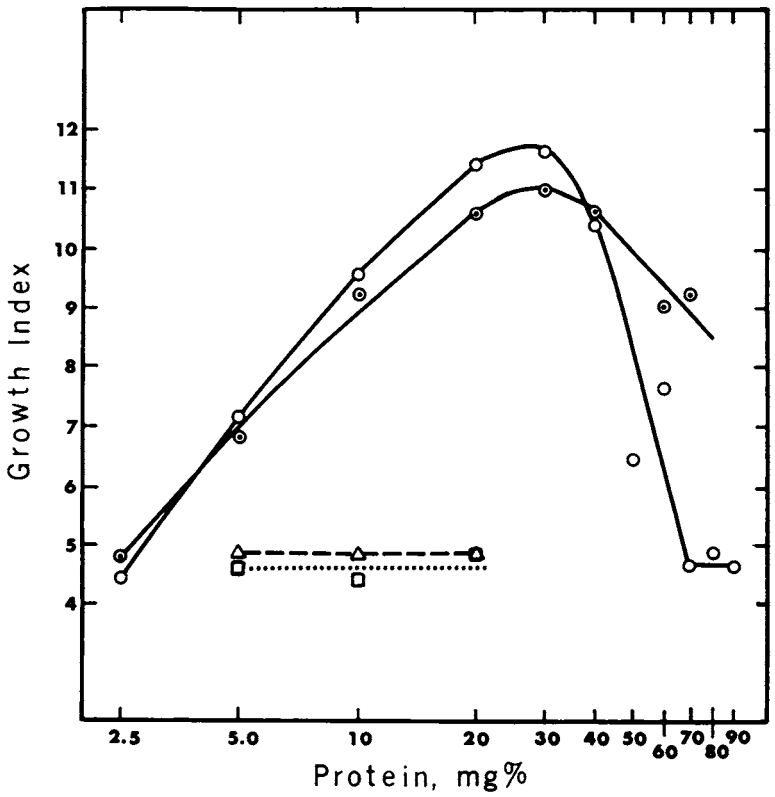

FIGURE 2. Relationships between constant particulate starch (100 mg%) and varying quantities of precipitated proteins. ○ = precipitated proteins (albumin 6 p + γ globulin 2 p + β lactoglobulin 2 p); exper. 152; 10 ml medium 91 (no amino acids); 5 animals; 12 days growth. △ = γ-globulin; exper. 152, same conditions. □ = β-lactoglobulin; exper. 152, same conditions. ⊙ = egg albumin; exper. 180; 10 ml medium 100 (no amino acids); 5 animals; 12 days growth. *Growth index*, see Material and Methods.

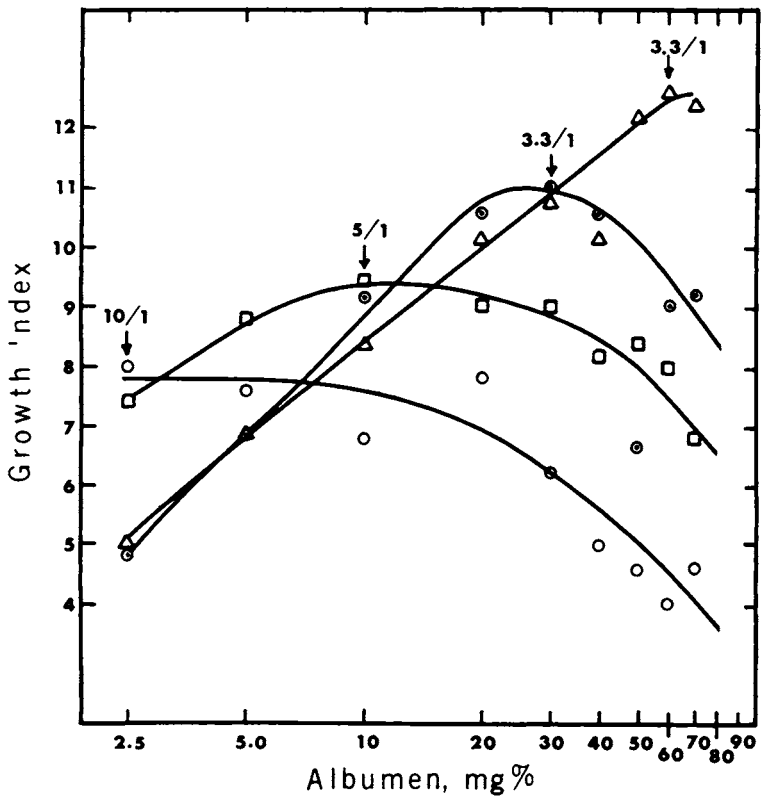

FIGURE 3. Relationship between 4 levels of particulate starch and varying quantities of precipitated albumin. △ = starch constant at 200 mg%. ⊙ = starch constant at 100 mg%. □ = starch constant at 50 mg%. ○ = starch constant at 25 mg%. Conditions: Exper. 180; 10 ml medium 100 (no amino acids); 5 animals; 12 days growth. ↓ = numbers stand for optimal starch/albumin ratio.

Phagotrophy and osmotrophy

Studies on rats and chickens have shown that for efficient diets the components must be balanced. Since in medium 39 the major components, carbohydrates and protein were particulate, it became necessary to vary their ratios and total concentrations to detect possible competition between them and to establish optimal conditions.

Starch:protein ratios To determine optimal ratios, the insoluble starch was kept at 0.1% while varying the protein from 1 to 100 mg%, hence the total concentrations in particles varied from 0.101–0.2%. Fig. 2 shows a typical run; as noted earlier, the growth index was the average growth per animal based on the growth achieved by 5 nauplii in 10 ml medium (see "materials and methods"). The nutritional value of the components of the protein mixture was analyzed. Albumin was the essential component even though the protein mix elicited slightly higher growth. Both became inhibitory above 0.04% and albumin was less inhibi-

tory than the protein mixture. The optimal starch:albumin ratios were between 5:1 and 2.5:1. Obviously development and growth in the suboptimal range (1–10 mg%) depended chiefly on the quantity of protein.

In another series of experiments starch was supplied at 4 levels,—0.025, 0.05, 0.1, and 0.2%—and albumin varied from 1 to 100 mg% (Fig. 3).

As the level of starch increased more albumin was needed for optimal growth and at the lower levels of starch, albumin became inhibitory confirming the need for a ratio starch:albumin somewhere between 5:1 and 3.3:1.

Evidently both ratios and total particles affected growth making it necessary to keep total particles constant while varying the starch protein ratios from 1:1 to 30:1. This was done by inoculating 5 individuals in 5 ml of liquid medium to

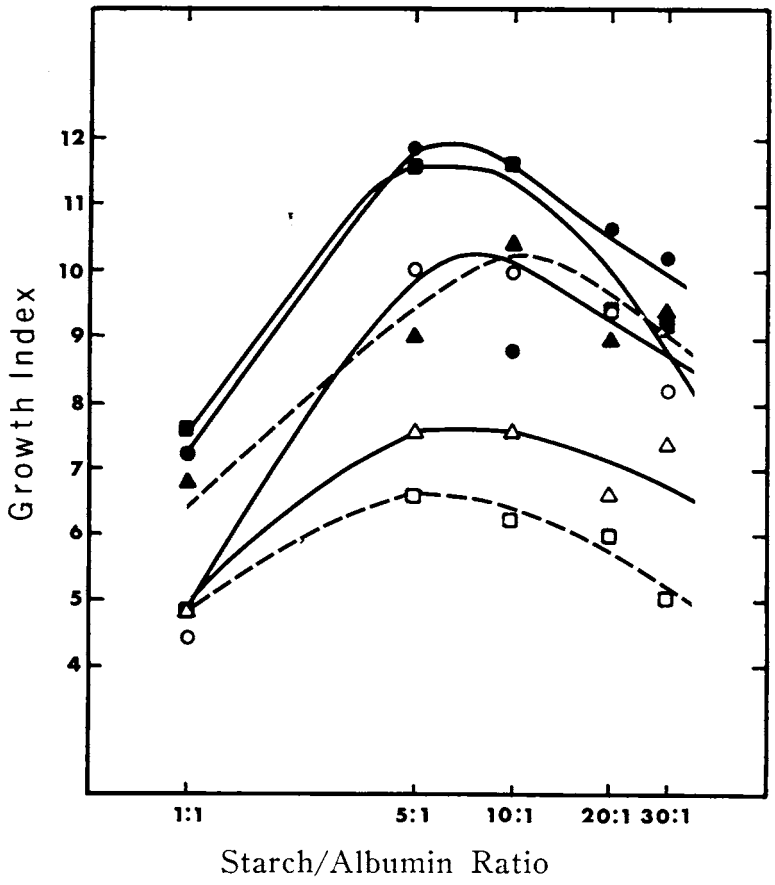

FIGURE 4. Relationships between 6 levels of total particles and varying starch per albumin ratios. Constant quantity of total particles: □ = 20 mg%; △ = 40 mg%; ○ = 80 mg%; ▲ = 160 mg%; ■ = 200 mg%; ● = 320 mg%. Conditions: Exper. 183; medium 100 (no amino acids); 5 animals in 6 ml medium; to final growth (*i.e.*, no time limit). Each point represents the average stage reached before dying by 5 animals when the total particles at its disposal were 1.2 mg (20 mg%); 2.4, 4.8, 9.6, 12, 19.2 mg.

which was added, 1 ml of the same medium, containing the desired ratio and amount of particles.

The growth index represented therefore the average growth per individual for only 6 ml medium (Fig. 4). As before the 1:1 ratio was inhibitory; the 5:1 and 10:1 ratios are optimal, and the 20:1 and 30:1 ratios are less effective. Obviously, the growth index at optimal ratios increases with the total particles offered, though the pairs 80 and 160 mg% and 200 and 320 mg% induce remarkably similar growth indexes.

As mentioned, these experiments had the objective of replacing dropwise feeding and to find ratios and total concentrations of the initial medium which would avoid mortality and permit high growth rates. Since these contrasting effects were favored by high quantities of particles, a compromise had to be made: 0.1% starch + 0.01–0.03% albumin proved satisfactory.

Replacing particles with solutes The particles employed were mixed polymers, obscuring the requirements for carbon sources and amino acids. Numerous trials to determine these requirements failed but contributed more understanding of phagotrophy and osmotrophy.

Repeated attempts to use soluble carbohydrates were done with media 39, 91 and 100. Starch was omitted, particulate proteins or albumin were used at 5–20 mg%. The carbohydrate solutions were filter-sterilized and/or autoclaved and added aseptically to the experimental test tubes. Soluble starch, dextrin, cellobiose, sucrose, galactose, trehalose, maltose, mannose, and glucose were tested in the range 0.4–4%.

Most failed to replace insoluble starch: one juvenile was obtained once in 1g% cellobiose, and a young female in 2% each of glucose + sucrose but in 3 months; a comparable female is obtained in 24–27 days on 0.1% insoluble starch. At best a 40× concentration of soluble sugars supported the same growth but in 3× the time needed for particulate starch.

From the early trials on replacing precipitated albumin with soluble mixtures of amino acids, it was evident that the amino-acid mixtures became toxic when > 0.4–0.6%, and that the nontoxic concentrations did not allow growth in the absence of precipitated protein or albumin. Since toxicity might be due to unbalances, many amino-acid mixtures were tried, including the mixtures employed for tissue cultures, for growing rats, chickens, insects, worms, and ciliates; also tried were mixtures simulating the amino-acid ratios of various proteins such as blood serum, liver, casein, albumin, globulins, and the protein composition of bacteria, yeast and algae.

These mixtures contained the 10 essential and the usual 8 nonessential. All of them became inhibitory or toxic at total concentrations of amino acids > 0.4–0.6%. [*Aedes aegypti,* a freshwater mosquito, tolerates up to 1.2% amino acid before inhibition ensues due to osmotic pressure. *Artemia* grows in 15% salts yet is sensitive to lower amino-acid concentrations.] Since at these concentrations the amino-acid mixtures could not substitute for all the albumin, further experiments were done in the presence of 2.5, 5, and 10 mg% precipitated albumin to detect partial substitution. Single amino acids and combinations of a few were also tried to find out whether albumin was deficient in any of them.

The 2 best amino-acid mixtures were AA 18L, and AA 19L (Table III): they partly replaced albumin if added to a medium 91 containing 0.1% starch + 5 mg%

TABLE III

Amino acids mixtures
(1 ml = mg)

Amino ac. mix / Compounds	AAP1	AAP2	AAP 18L	AAP 19L	AA 1A
L-arginine	2.6	3.0	4.6	5.7	
L-histidine	0.6		2.1	2.4	6.0
L-isoleucine	1.2		5.3	7.0	
L-leucine	1.6		9.7	9.2	
L-methionine	0.6	1.0	3.2	5.2	
L-lysine	1.4	1.0	7.6	6.3	
L-phenylalanine	1.2		5.3	7.7	3.0
L-threonine	1.0		5.1	4.0	6.0
L-tryptophane	0.3		1.6	1.2	
L-tyrosine	0.8	1.0	3.8	3.7	
L-valine	1.2		7.2	7.1	
L-cystine	0.6		2.7	2.4	
L-serine	0.6	2.0	7.9	8.2	12.0
L-proline	0.2	7.0	5.0	3.6	
L-glutamic ac.	0.6	30.0	13.5	16.5	30.0
L-alanine		15.0	5.0	6.7	
L-aspartic ac.			9.5	9.3	
glycine	0.6	20.0		3.1	
Total weight/ml	15.1	80.0	99.1	109.3	57.0
ml/100 used	5 ml	AAP_1 6 ml +AAP_2 1 ml	6–9 ml	3–6 ml	3.3 ml
Total amino ac.	75.5 ml	171 mg	594–891 mg	328–656 mg	188 mg
Used in medium	31	39	91	91	100

albumin: 0.3% of AA 18L replaced 3–5 mg% albumin, giving in the same time similar development. AA 19L was better: 0.33% substituted 5–8 mg% of albumin, *i.e.*, gave growth comparable to albumin 10–13 mg%. Therefore, 0.3% of soluble amino acids correspond in nutritional efficiency to 5 mg of precipitated albumin, *i.e.*, particles were ~60× more efficient than solutes.

But additions of amino-acid mixtures to a medium containing 0.1 starch + 2.5 mg% albumin stimulated growth only very slightly. This is the minimal quantity of albumin which gave some growth with or without amino acids. The complete substitution of particulates with glucose + sucrose + amino acids also resulted in no growth, with or without non-nutrient particles (see below).

However, the addition of a few amino acids (AAIA mix, Table III) to medium 100 speeded growth and gave better survival, indicating that albumin may be deficient in some amino acids.

Uptake of soluble nutrients. Failure to replace particulate carbohydrates and protein indicated strongly that *Artemia* was an obligate phagotroph. Yet paradoxically, the B-vitamins, nucleic acid components, and trace metals were offered as solutes. Still, these solutes might have become absorbed on to the particles.

This possibility was explored by preparing 2 batches of medium 91: one without vitamins, the other with vitamins (2 ml% Vit. A. VIII). The two batches were distributed in test tubes, autoclaved and centrifuged until all the particulate matter was well packed.

Both supernatants were withdrawn aseptically and passed through an ultra-fine sterile glass filter. The 2 filtrates and the 2 slurries were combined aseptically to yield all 4 possible combinations (10 ml per tube); each was inoculated with 5 nauplii.

	stage reached
1) Filtrate and particles from *no* vitamins	IV small metanauplii
2) Filtrate *no* vitamins + particles vitamins	IV medium metanauplii
3) Filtrate vitamins + particles *no* vitamins	juveniles
4) Filtrate and particles from vitamins	young adults

The results indicate that most of the vitamins remained in the filtrate and that the particles absorbed little or no vitamins. The slight difference in growth between the pairs 1, 2 and 3, 4 which might be interpreted as a very small absorption of vitamins on the particles, was probably due to the residual liquid medium which mixed with the particles in decanting.

This simple experiment resolves the apparent paradox of an animal which utilizes micronutrients as solutes yet needs bulk nutrients as particles. Inbibition occurs in *Artemia* but is so limited that only the nutrients needed as traces, and nontoxic when supplied in enormous excess, are effective in soluble form.

Effects of non-nutrient particles Several particles were tried in the hope of increasing liquid ingestion. These were tried on "juveniles" in seawater and on "young" in artificial media without starch and precipitated protein but rich in amino acids and sugars. All particles before use were ground in a colloid mill to an ingestible range (1–20 μ); all were ingested as attested by numerous fecal pellets.

Survival of "juveniles" and "young" varied depending on the particle: early mortality (*e.g.*, 8 days in seawater, 10 days in artificial media) ensued in glass, vegetable charcoal, Celite, montmorillite, graphite, Alfacell and Celloflour. Maximal survivial ((11 days in seawater, 20 days in artificial media) was induced by kaoline, Carbose, and rice starch. Cellulose, activated charcoal, and alumina gave average survival. Although 2–3 molts occurred in artificial media with the more favorable particles, the "young" did not become "young adults" except in tubes containing rice starch. The favorable action of these particles in artificial media was merely longer survival. Adsorbant particles were also tried, among them Norit A, Sephadex, and a fine powder of mixed weakly cationic and anionic resins; they were inhibitory.

Reilly (1964) reported success in eliminating the apparent requirement of *Paramecium caudatum* for the "protein factor" (a non-dialyzable fraction from dried green peas) by adding Celkate (anhydrous Mg silicate, Johns-Manville, New York) to a conventional synthetic *P. caudatum* medium to which had been added larger quantities of serine. The "protein factor" apparently both stimulated vacuole formation (the main mechanism for ingesting and digesting particles in most ciliates) and supplied amino acids suboptimal in previous media. Norit A and various starch particles were active but less effective than Celkate for *P. cau-*

datum. Adsorptive particles were also beneficial for *Glaucoma chattoni* (Holz, Wagner, Erwin and Kessler, 1961).

Celkate and Micro-cel (a hydrous Ca silicate) were tried in the artificial media containing a total of 0.72% of amino acids + 0.05% starch and 5 mg% of precipitated albumin. Celkate, although inhibitory above 10 mg%, somewhat favored growth and differentiation, giving in general one stage above the controls.

Discussion and Conclusions

The central problem in designing media was how to supply nutrients effectively. Medium 100 is almost defined; it embodies what was found about *Artemia's* needs for B vitamins, sterols, and nucleic acid derivatives. Details of these requirements will be reported separately.

Requirements for individual amino acids and carbohydrates could not be determined because the precipitated albumin and starch could not be replaced with their water-soluble ingredients. The vitamins and nucleic acids in contrast were effective as solutes.

Evidently the need for building blocks (amino acids) and energy sources—the bulk of the requirements—could not be satisfied as solutes.

These results and the experiments on the protein: starch ratios and on non-nutrient particles agree with most of the observations on the physiological behavior of *Artemia,* and indicate that it is an obligate phagotroph.

Artemia discriminates only for size and does not select from various mixed suspension of *Phaeodactylum, Chlorella, Dunaliella,* and *Arteromonas;* nor does it discriminate between plant cells and sand (Reeve, 1963b).

Indiscriminate uptake of particles may handicap phagotrophs. Growth of *Daphnia* may be impaired by non-nutritive particles in lakes turbid with fine particles of clay and silt. In trial of various mixtures of inert particles and algae, Robinson (1957) found that survival and reproduction increased at very low concentrations of inert particles, but declined as the abundance of mineral particles increased.

Equally important are variations in nutrient value of ingested algae even among species of the same genus, (from inadequate for growth, to partly or completely adequate); the same algal species may be adequate for one crustacean species, inadequate for another (Provasoli, Shiraishi and Lance, 1959). It is, however, this lack of discrimination that permitted replacement of live food with artificial particles of acceptable size.

Reeve (1963a) found also that *Artemia* regulates food intake by increasing ingestion rates as cell concentrations increase until a certain concentration of cells is reached; at this concentration, or above, a constant, maximum ingestion rate is maintained [*Daphnia magna* behaves similarly (McMahon and Rigler, 1965)]. In our experiments the concentration of particles was far above those used by Reeve, even at the reduced concentrations of particulates of medium 100, therefore *Artemia* was ingesting at a constant maximum rate.

Maximum ingestion rate is inversely proportional to algal cell size, thus the total volume of cells ingested at this rate remained the same for a given size of animal: 0.05 mm^3/hr/10 mm long adult (Reeve, 1963a). Hence the size varia-

bility of the artificial particles, in media 39, 91, and 100 was not important, since it was within limits of acceptability.

The difficulty in compounding artificial particulate media derives from the evolutionary adaptions of *Artemia*. Maintenance of a constant rate of ingestion in conditions of overabundance of particles, reflects the need to retain the food for the time necessary for digestion; lacking this regulation over-feeding could result in starvation due to inadequate retention time. But the retention time of *Artemia* is probably attuned to the ingestion of living cells which, upon digestion of the cell walls, become a fine suspension of organelles, colloids, and solutes—all rapidly digestible because of the enormous surface of the particles. Ingestion of live cells may be an added advantage to an organism living like *Artemia* in a hypersaline environment: the cells provide, besides nutrients, 85–90% water. *Artemia* is excellently fit to its environment: most of the few algal species living in brines are a complete food for *Artemia* (Gibor, 1956). That the retention time in the gut during maximum feeding rates is very close to the minimum time needed for digesting cells with a thin cell wall is shown by the lack of nutrient value of algae (as *Stichococcus*) which, having a thicker and probably a less digestible cell wall, pass through the gut of *Artemia* undigested and alive (Gibor, 1956).

Since Reeve (1962 *in* 1963b, p. 141) asserts that "food remains in the gut progressively longer, as there is less pressure on it from the incoming food to move through the gut," it would have been better to use in artificial media more dilute suspensions of particles: a longer stay in the gut would permit better digestion. Dropwise feeding gave faster growth, perhaps because of the far lower concentration in particles but, as mentioned, it was too laborious for aseptic work. Similarly, less particles were not employed in medium 100 because more tubes of media would have been needed to bring a few individuals to adulthood.

That time of retention, nature of particle, and limited size of the gut govern *Artemia* nutrition is indicated by the experiments with various ratios of precipitated starch and protein. Overabundance of either meant less growth or no growth (where proteins predominated), even though both particles were needed. If nutrient particles can compete for the available space of the gut, it is not surprising that non-nutritive absorbent particles had no effect on growth even though high concentrations of sugar and/or amino acids were present. Evidently very small (ground with a colloidal mill) absorbent particles, even if fully charged with nutrients, could not approach the effect on growth of like-size nutrient particles whose core and surface both were nutritive. Particles $< 1\ \mu$, or colloids undoubtedly would favor digestive efficiency (as happens when the cell wall of an alga is broken) but this was impractical because a minimal particle size is needed for full efficiency of the paropodia in sweeping the medium clear of particles and collecting them on the food groove as ingestible pellets.

Is, then, *Artemia* an obligate phagotroph devoid of osmotrophy? Since the 1959 medium provided all nutrients except starch, in soluble form we said (Provasoli and Shiraishi, 1959, p. 354) it utilized solutes for growth. We had not considered that serum is a colloid and that it could have been absorbed on to the starch particles. This conclusion was also influenced by Croghan (1958a–bcd). By ingenious, painstaking methods he found that *Artemia* regulates the osmotic pressure and ionic balance of its hemolymph by taking up NaCl and water from

the gut lumen and excreting NaCl through the branchiae (metepipodites) of the first 10 pair of phyllopods (*i.e.,* swimming appendages)—the only permeable region of the whole exoskeleton.

Croghan (1958d) also kept adult *Artemia* 24 hr in seawater with dissolved neutral red. The dye rapidly colored the gut lumen, did not diffuse in the hemolymph, and became 7× more concentrated in the gut than in the surrounding medium. He concluded (p. 244) that ". . . *Artemia* rapidly swallows the medium and takes up water from it, thus concentrating the dye." The experiment of Croghan was repeatable, and allowed, in the presence or absence of particulate starch, a determination of the approximate pH of the various sections of the gut: 5.6–6.0 in the cephalic region; 6.0, 6.6, 7.0 in the thoracic region; the abdominal region remained colorless.

Our experiment showing that the B vitamins in the medium are not absorbed onto the particles and are used as solutes substantiates the conclusion that *Artemia* swallows the medium. But swallowing of medium should be minor: *a*) mixtures of sugars and complete amino acid mixtures were very poorly utilized; *b*) rapid or substantial swallowing of the highly hypersaline waters (up to 6× seawater) in which *Artemia* normally lives would impose severe stress for maintaining the hemolymph below or at the highest osmotic pressure allowable (corresponding to ~2.2% NaCl).

We must conclude that osmotrophy in *Artemia* is quite limited, and by itself incapable of sustaining growth even at concentrations of nutrients approaching inhibitory levels. In nature *Artemia* lives as an obligate phagotroph but in the laboratory it can profit in extreme conditions (very high concentrations of vitamins and nucleic acids) from its modest osmotrophy. This view is supported by the finding of Stephens and Schinske (1961) that marine Crustacea differ from all soft-bodied invertebrates in having almost no uptake of radioactive glycine.

So far, the only filter-feeders reared on artificial media are 3 mosquitoes; most work was done on *Aedes aegypti.* Singh and Brown (1957) grew it on a synthetic diet in which amino acids, RNA, vitamins, and glucose were added as solutes; the solid portion was constituted of 0.63% of finely powdered cellulose coated with a mixture of lecithin, cephalin, and cholesterol. The mixture of 17 L-amino acids was added at 1.16%. Higher concentrations were inhibitory because they exceeded *A. aegypti* osmotic tolerance (Δ 0.4 C). [The inhibitory level of the amino-acid mixtures for *Artemia* is 0.7% while the osmotic tolerance is 30% total salts!] If the amino-acid mixture was added at 0.8%, only 15% of the mosquito larvae reached the 3rd instar and none the 4th instar, indicating that even in *A. aegypti* solute efficiency is low. Also, the larval period lasted 16 days when the larvae were grown on 1.16% of the amino acid mixture (mimicking casein ratios) as compared with 7.5 days on the corresponding diet with casein.

Akov (1962), in studying antimetabolites, resumed work on *A. aegypti.* Under her conditions she found that the amino-acid mixture of Singh and Brown was extremely inefficient [median time to pupation (MTP) was 45 days; only 15% became adults.] If 0.05% of finely powdered (80 mesh) dry-sterilized casein was added to the amino-acid mixture, 95% of the larvae reached adults; the MTP was 10 days. The inefficiency of the solutes was clearly shown when casein hydrolysate was compared with solid casein: with 2% of the hydrolysate 12 days were

needed for pupation and 60% of the larvae became adults; with 0.1% casein, median time to pupation was 6.5 days and 90% adults were obtained!

Thus even for *A aegypti,* a filter-feeder which can be grown on solutes, phagotrophy is by far the most efficient source of building blocks and energy.

An important difference between *Artemia* and insects is that casein (or amino-acid mixtures mimicking it) seem well balanced for all insects so far investigated, while it is ineffective or toxic for *Artemia* when powdered and dry-heat sterilized; several samples were tried with similar results. Many other proteins besides egg albumin were dissolved and heat precipitated at their isoelectric point, but unfortunately these particulates redissolved when autoclaved in the alkaline medium needed by *Artemia* (*Artemia* cannot grow below below pH 6.8 in seawater or our artificial seawater media).

Another difference is that *A. aegypti* did not need any carbohydrate when the solid casein was supplied at 1%. *Artemia,* on the contrary, needed ratios of 10:1–2.5:1 for starch-albumin; albumin alone was toxic. This need for a preponderance of starch could be an artifact since the composition of 10 marine algae tested by Parsons, Stephens and Strickland, (1961) is similar: carbohydrates 15–30%, proteins 36–57%, fats 3–10%. It might however be an Artemia peculiarity since *Daphnia magna* raised on similar artificial media likes a predominance of protein.

Preliminary work on *D. magna* indicates also that the particulates are as highly efficient as in *Artemia. Daphnia* was brought in aseptic culture as a comparative organism for deciding whether phagotrophy in *Artemia* was not due to its need to avoid swallowing hypersaline media. *Daphnia,* living in fresh waters, has no need to avoid swallowing, and being able to grow in highly organic solutions of dung (Banta medium) should be more resistant to organics.

Phagotrophy being the most efficient system, the problem of designing artificial media for filter-feeders centers in supplying all the nutrients, so far as possible, as particulates.

Summary

1. The 1959 undefined artificial medium for *Artemia* was simplified to a medium containing only defined ingredients: a liquid phase containing mineral salts, 6 amino acids, 5 nucleic acid components, 8 vitamins, 2 sugars, a pH buffer, and a fine particulate phase consisting of precipitated albumin, gelled rice starch, and cholesterol. The amino acids and sugars are dispensible.

2. Starch and albumin were not replaceable by their soluble components (sugar and amino acids) even in the presence of inert or absorbing particles. Phagotrophy appeared the most efficient way to satisfy the bulk nutritional requirements.

3. Growth rate and differentiation depended upon starch:protein ratio and total quantity of particles.

4. *Artemia* ingested liquids but apparently to a very limited extent since vitamins and nucleic acid components (nontoxic even at very high concentrations) were utilized as solutes only when in high concentrations. Amino acid mixtures on the other hand became toxic at concentrations too low to satisfy growth requirements.

LITERATURE CITED

AKOV, S., 1962. A qualitative and quantitative study of the nutritional requirements of *Aedes Aegypti* L. larvae. *J. Insect Physiol.*, **8**: 319–335.

CROGHAN, P. C., 1958a. The survival of *Artemia salina* (L.) in various media. *J. Exp. Biol.*, **35**: 213–218.

CROGHAN, P. C., 1958b. The osmotic and ionic regulation of *Artemia salina* (L.) *J. Exp. Biol.*, **35**: 219–233.

CROGHAN, P. C., 1958c. The mechanism of osmotic regulation in *Artemia salina* (L.): The physiology of the branchiae. *J. Exp. Biol.*, **35**: 234–242.

CROGHAN, P. C., 1958d. The mechanism of osmotic regulation in *Artemia salina* (L.): The physiology of the gut. *J. Exp. Biol.*, **35**: 243–249.

D'Agostino, A., 1965. Comparative studies of *Artemia salina* (development and physiology). *Ph.D. thesis, New York University,* 82 pp.

GIBOR, A., 1956. Some ecological relationships between phyto- and zooplankton. *Biol. Bull.*, **111**: 230–234.

HEATH, H., 1924. The external development of certain Phyllopods. *J. Morphol.* **38**: 453–483.

HOLZ, G. G., B. WAGNER, J. ERWIN, AND D. KESSLER, 1961. The nutrition of *Glaucoma chattoni*, A. *J. Protozool.*, **8**: 192–199.

LILLY, D. M. AND R. C. KLOSEK, 1961. A protein factor in the nutrition of *Paramecium candatum. J. Gen. Microbiol.*, **24**: 327–334.

MCMAHON, J. W. AND F. H. RIGLER, 1965. Feeding rate of *Daphnia magna* Strauss in different foods labeled with radioactive phosphorus. *Limnol. Oceanogr.*, **10**: 105–113.

PARSONS, T. R., K. STEPHENS AND J. D. H. STRICKLAND, 1961. On the chemical composition of eleven species of marine phytoplankters. *J. Fish. Res. Board Canada,* **18**: 1001–1016.

PROVASOLI, L. AND A. D'AGOSTINO, 1962. Vitamin requirements of *Artemia salina* in aseptic culture. *Amer. Zool.*, **2**: 439.

PROVASOLI, L., J. J. A. MCLAUGHLIN AND M. R. DROOP, 1957. The development of artificial media for marine algae. *Arch. Mikrobiol.*, **25**: 392–428.

PROVASOLI, L. AND K. SHIRAISHI, 1959. Anexic cultivation of the brine shrimp *Artemia salina. Biol. Bull.*, **117**: 347–355.

PROVASOLI, L., K. SHIRAISHI AND J. R. LANCE, 1959. Nutritional idiosyncrasies of *Artemia* and *Tigriopus* in monoxenic culture. *Ann. New York Acad. Sci.*, **77**: 250–261.

REEVE, M. R., 1963a. The filter-feeding of *Artemia*. I. In pure cultures of plant cells. *J. Exp. Biol.*, **40**: 195–205.

REEVE, M. R., 1963b. The filter-feeding of *Artemia*. II. In suspensions of various particles. *J. Exp. Biol.*, **40**: 207–214.

REEVE, M. R., 1963c. Growth efficiency in *Artemia* under laboratory conditions. *Biol. Bull.*, **125**: 133–145.

REILLY, M., 1964. Importance of adsorbents in the nutrition of *Paramecium caudatum. J. Protozool.*, **11**: 109–113.

ROBINSON, M., 1957. The effects of suspended materials on the reproductive rate of *Daphnia magna. Publ. Inst. Mar. Sci. Univ. Tex.*, **4**: 265–277.

SHORB, M. S. AND P. G. LUND, 1959. Requirement of Trichomonads for unidentified growth factors, saturated and unsaturated fatty acids. *J. Protozool.*, **6**: 122–130.

SINGH, K. R. P. AND A. W. A. BROWN, 1957. Nutritional requirements of *Aedes aegypti* L. *J. Insect Physiol.*, **1**: 199–220.

STEPHENS, G. C. AND R. A. SCHINSKE, 1961. Uptake of amino acids by marine invertebrates. *Limnol. Oceanogr.*, **6**: 175–181.